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(54) **Title:** METHODS FOR EVALUATING PATHOLOGIC CONDITIONS USING EXTRACELLULAR RNA

(57) **Abstract:** This invention provides methods for the detection, diagnosing, monitoring or predicting of non-neoplastic diseases, pathologic conditions, and injury. The methods of the invention detect extracellular non-neoplastic mammalian RNA in the blood, blood plasma, serum, or other bodily fluid of an animal, most preferably a human, having or predisposed to having a non-neoplastic disease, pathologic condition, or injury.

METHODS FOR EVALUATING PATHOLOGIC CONDITIONS USING EXTRACELLULAR RNA

This application claims priority to U.S. Provisional Patent Application Serial No. 60/308,054, filed July 25, 2001, the disclosure of which is specifically incorporated by reference herein.

BACKGROUND OF THE INVENTION

This invention relates to methods for diagnosing, detecting, evaluating, and monitoring non-neoplastic pathologic conditions and diseases within an animal, preferably a human. Said pathologic conditions and diseases include, *inter alia*, pathologic conditions and diseases affecting specific body organs and those affecting multiple organs or bodily organ systems, and those pathologic conditions that are associated with disease or injury, or that are predictive for a disease or that can ultimately result in a disease. As set forth herein, the invention provides methods for detecting mammalian ribonucleic acid (RNA) in said animal's blood plasma, serum, or other bodily fluid. The methods of the invention thereby enable evaluation of gene expression that is associated with, consequent to, or predictive of pathologic conditions and diseases or cellular injury and trauma. The invention also provides methods that permit cellular response and recovery to pathologic conditions and disease as well as cellular injury to be monitored. The invention thereby provides methods for evaluating and monitoring response to specific therapies for said pathologic conditions, diseases, and injuries. The invention also specifically provides methods for evaluating and monitoring non-hematopoietic or non-hematological cells and tissues that are terminally differentiated. In these methods, extracellular RNA derived from said cells and tissues is detected in a

bodily fluid. The invention also permits the diagnosing, detecting, evaluating, and/or monitoring of pathologic conditions and diseases affecting non-proliferating cells and tissues, such as those of the heart, brain and muscle. The invention is thus particularly advantageous for evaluating pathologic conditions and diseases of the cardiovascular system, the nervous system and of skeletal muscles. The invention further allows the detection of non-neoplastic cells and tissues that are proliferating normally or consequent to disease or injury. The invention permits detection of extracellular mammalian RNA associated with non-neoplastic disease that is not transcribed from a fragile site or does not contain viral or bacterial nucleic acid sequences.

While the etiologies of non-neoplastic pathologic conditions and diseases are varied, the pathologic process is often characteristically associated with intracellular production or over-production, or escape or release, of specific proteins from the cell that can characterize the cell. Such proteins may be involved in cellular adaptive responses, or be indicative of cellular injury, or reflect the production of proteins associated with the disease state itself. Furthermore, proteins normally associated with a cell or tissue's metabolism may be overproduced within a cell, or be secreted from the cell, or be inappropriately released from the cell. In clinical practice, detection of proteins in blood and other bodily fluids has been utilized in the diagnosis and monitoring of disease. However, not all such proteins may be detectable in blood or bodily fluids, often because the protein is either not secreted or released from the cell, or exists in blood at levels below limits of detection for a given stage of disease, particularly at early or subclinical stages of disease. There thus exists a need for new methods

that provide for the analysis of cellular gene expression in a more sensitive manner.

Ribonucleic acid (RNA) is essential for producing cellular proteins, and detecting and monitoring mammalian RNA can be used to assess cellular gene expression. Furthermore, since RNA and deoxyribonucleic acid (DNA) can be hybridized and amplified in a qualitative or quantitative manner using nucleic acid amplification methods, detection of RNA can be performed with high sensitivity. Although the prior art contained sporadic reports suggesting that RNA might be detected in plasma and serum (*e.g.*, Wieczorek *et al.*, 1985 *Proc Natl Acad Sci USA* 82: 3455-3459; Wieczorek *et al.*, 1987 *Cancer Res.* 47: 6407-6412; Wieczorek *et al.*, 1989 *Schweiz med Wschr* 119: 1342-1343; Kamm and Smith, 1972 *Clin. Chem.* 18: 519-522), until recently it was unknown whether specific RNA species existed in plasma or serum with sufficient integrity to be amplified and detected. Co-owned U.S. Patent No. 6,329,179 B1, incorporated herein by reference in their entirety, provide methods for detecting extracellular tumor RNA in blood plasma, serum, and bodily fluids. After the priority date of the co-owned application, several authors have confirmed that tumor RNA can be amplified from plasma or serum (Kopreski *et al.*, 1999 *Clin. Cancer Res.* 5: 1961-1965; Chen *et al.*, 2000 *Clin. Cancer Res.* 6: 3823-3826; Dasi *et al.*, 2001 *Lab. Invest.* 81: 767-769; Hasselmann *et al.*, 2001 *Oncology Reports* 8: 115-118; Kopreski *et al.*, 2001 *Clin. Chem.* 47: 362, abstract 9; Fleishhacker *et al.*, 2001 *Clin. Chem.* 47: 369, abstract 48; Reinhold *et al.*, 2001 *Clin. Chem.* 47: 369, abstract 50; Gocke *et al.*, 2001 *Clin. Chem.* 47: 369, abstract 51), and further that fetal RNA is detectable in maternal plasma (Poon *et al.*, 2001 *Clin. Chem.* 47: 363, abstract 11). These findings are notable since it is well established in the art that ribonucleases present

in blood rapidly degrade mammalian RNA (Reddi and Holland, 1976 *Proc Nat Acad Sci USA* 73: 2308-2310), and further that one consequently can not amplify free RNA from plasma or serum following cellular lysis (Komeda *et al.*, 1995 *Cancer* 75: 2214-2219; Pfleiderer *et al.*, 1995 *Int. J. Cancer* 64: 135-139). Mammalian RNA has also been demonstrated in sera in association with viral nucleic acid, and fragile sites, such as in association with hematologic cancer cells (Urnovitz *et al.*, 1999 *Clin. Diag. Lab. Immunology* 6: 330-335; Urnovitz, U.S. Patent Serial No. 6,344,317). Since the etiology and physiology of extracellular RNA remains unknown, detection of extracellular RNA in non-virally mediated, non-neoplastic disease processes, and particularly from non-hematological cells and tissues that include non-proliferating tissues and terminally differentiated cells and tissues of diseased or injured solid organs, was both unknown and unexpected.

Neoplasia is characterized by pathophysiologic processes that often differ from those of non-neoplastic disease. Similarly, fetal development may be viewed as a proliferative process of cells undergoing differentiation characterized by physiologic processes that often differ from those occurring in non-neoplastic disease. It was unknown in the art that extracellular mammalian RNA derived from non-neoplastic solid organ tissue could be detected in the blood plasma, serum, or other bodily fluids of individuals with disease at levels higher than present in the blood plasma or serum or bodily fluid of healthy individuals. This is particularly true for non-neoplastic, non-virally mediated RNA specific to the non-proliferating, terminally differentiated non-hematopoietic or non-hematological cells and tissues of the heart and brain.

SUMMARY OF THE INVENTION

The present invention provides methods for diagnosing, evaluating, monitoring or predicting within an animal, most preferably a human, the existence of a non-neoplastic disease or pathologic condition or injury. In preferred embodiments, the method comprises the step of detecting extracellular mammalian RNA in a bodily fluid of an animal, preferably blood and most preferably blood plasma or serum, urine, effusions, ascites, saliva, cerebrospinal fluid, cervical secretions, vaginal secretions, endometrial secretions, gastrointestinal secretions, sputum and bronchial secretions, and/or associated lavages, wherein said RNA is present in the bodily fluid of an animal with a non-neoplastic disease or pathologic condition or cellular injury, and not present in the bodily fluid of a healthy animal, or wherein said RNA is present in a bodily fluid of an animal with a non-neoplastic disease or pathologic condition or cellular injury in quantitative amounts that are greater than are present in the bodily fluid of a healthy animal.

The invention provides methods for amplifying and detecting extracellular mammalian RNA associated with non-neoplastic disease in blood, more preferably in blood plasma or serum, or in other bodily fluids, the method comprising the steps of extracting RNA from said bodily fluid, *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA derived therefrom, either qualitatively or quantitatively, and detecting the amplified product or signal produced thereby.

In a preferred embodiment, the RNA is derived from a non-hematopoietic or non-hematological cell or tissue. In one aspect of this embodiment, the RNA is derived from a non-proliferating cell or tissue. In a second aspect, the RNA is derived from a terminally-differentiated cell or tissue. In a third aspect, the RNA

does not contain viral nucleic acid sequences. In a fourth aspect, the RNA is not derived from transcription of a fragile site.

The invention further provides methods for detecting organ-specific or tissue-specific extracellular mammalian RNA present in plasma, serum, and/or other bodily fluid by hybridization, wherein the RNA is derived from specific non-neoplastic non-hematopoietic or non-hematological cells and/or tissue from an animal, most preferably a human, or cDNA derived therefrom, to a specific primer, probe, solid substrate or bioelectrical interface, the method comprising the steps of extracting RNA from said bodily fluid, and hybridizing a portion of the extracted RNA or cDNA derived therefrom to a specific primer, probe, solid substrate, or bioelectrical interface consisting of oligonucleotide sequences complimentary to RNA or cognate cDNA from specific, non-neoplastic, non-hematopoietic or non-hematologic cells and/or tissue.

In preferred embodiments, the extracellular RNA detected using the methods of this invention are not amplified. In one aspect of this embodiment, the RNA does not contain viral nucleic acid sequences. In a second aspect of this embodiment, the RNA is not transcribed from a fragile site. In a third aspect of this embodiment, the cells or tissues are terminally differentiated. In a fourth aspect of this embodiment, the cells or tissues are non-proliferating.

The invention further provides methods for detecting and monitoring mammalian RNA or cDNA derived therefrom, in blood, preferably blood plasma, serum, and other bodily fluids from an animal, most preferably a human, that is associated with non-hematopoietic or non-hematological cells or tissue affected by a non-neoplastic disease or injury, wherein the method comprises the steps of extracting RNA from said bodily fluid, *in vitro* amplifying or signal amplifying a

fraction of the extracted RNA or cDNA derived therefrom, either qualitatively or quantitatively, and detecting the amplified product or signal produced thereby.

In one aspect of this embodiment, the RNA does not contain viral or retroviral nucleic acid sequences. In a second aspect of this embodiment, the RNA is not transcribed from a fragile site. In a third aspect of this embodiment, the cells or tissues are terminally differentiated. In a fourth aspect of this embodiment, the cells or tissues are non-proliferating.

The invention further provides methods for detecting and monitoring mammalian RNA or cDNA derived therefrom, in blood, preferably blood plasma or serum, or other bodily fluid from an animal, most preferably a human, that is derived from non-neoplastic cells or tissues, wherein said mRNA produces a protein that has a consequent deleterious effect upon other differing non-neoplastic cells or tissues, thereby resulting in a disease or pathologic condition of the cells or tissues or their organ(s) and organ system(s) thereby deleteriously affected. In this embodiment, the method comprises the steps of extracting RNA from blood plasma, serum, or other bodily fluid, *in vitro* amplifying or signal amplifying the RNA or cDNA derived therefrom, either qualitatively or quantitatively, and detecting the amplified product or signal produced thereby.

In one aspect of this embodiment, the RNA does not contain viral or retroviral nucleic acid sequences. In another aspect of this embodiment, the RNA is not transcribed from a fragile site. In another aspect of this embodiment, the cells or tissues are terminally differentiated. In another aspect of this embodiment, the cells or tissues are non-proliferating.

The invention further provides methods for detecting and monitoring mammalian RNA or cDNA derived therefrom, in blood, most preferably plasma or

serum, and/or other bodily fluid from an animal, most preferably a human, that is associated with non-neoplastic, terminally differentiated non-hematopoietic or non-hematological cells or tissues, including either healthy or diseased tissues, the method comprising the steps of extracting RNA from said bodily fluid, *in vitro* amplifying or signal amplifying the extracted RNA or cDNA derived therefrom either qualitatively or quantitatively, and detecting the amplified product or signal produced thereby.

In one aspect of this embodiment, the present invention provides methods for detecting mammalian RNA associated with non-hematopoietic or non-hematological cells and tissues that are characteristic of specific tissue(s) or organ(s) or organ system(s), either diseased or healthy. In this aspect, the methods of the invention comprise the steps of extracting RNA from blood, most preferably blood plasma or serum, or other bodily fluid, *in vitro* amplifying or signal amplifying RNA comprising said extracted RNA or cDNA derived therefrom, associated with non-hematological cells and tissues of specific organ(s) or organ system(s), either qualitatively or quantitatively, and then detecting the amplified product or signal. In a particularly preferred embodiment, the cells and tissues are those of the heart or cardiovascular system. In another particularly preferred embodiment, the cells and tissues are those of the brain or nervous system. In other particularly preferred embodiments, the cells and tissues are those of the gastrointestinal system, endocrine system, genitourinary system, respiratory system, musculoskeletal system, or skin.

In a second aspect of this embodiment, the invention provides methods for detecting mammalian RNA from a non-hematopoietic or non-hematological, non-proliferative tissue in a bodily fluid such as blood, blood plasma, serum, or

cerebrospinal fluid. In this aspect, the methods of the invention comprise the steps of extracting mammalian RNA from said bodily fluid, *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA derived therefrom, comprising said extracted RNA associated with a non-hematopoietic or non-hematological non-proliferative tissue, either qualitatively or quantitatively, and then detecting the amplified product or signal thereby. In a particularly preferred embodiment, the non-proliferative tissue is heart tissue, preferably cardiac muscle tissue. In another particularly preferred embodiment, the non-proliferative tissue is brain tissue, preferably neural tissue.

In preferred embodiments of the inventive methods, extracellular mammalian RNA is extracted from a bodily fluid such as whole blood, blood plasma or serum, or cerebrospinal fluid, using an extraction method such as gelatin extraction method; silica, glass bead, or diatom extraction method; guanidinium thiocyanate acid-phenol based extraction methods; guanidinium thiocyanate acid based extraction methods; methods using centrifugation through cesium chloride or similar gradients; phenol-chloroform based extraction methods; or other commercially available RNA extraction methods. Extraction may further be performed using probes that specifically hybridize to specific RNA, including probes attached to solid substrates or to magnetic beads or similar particles.

In preferred embodiments of the inventive methods, mammalian RNA or cDNA derived therefrom, or a signal derived therefrom, is amplified using an amplification method such as reverse transcriptase polymerase chain reaction (RT-PCR); ligase chain reaction; DNA signal amplification; amplifiable RNA reporters; Q-beta replication; transcription-based amplification; isothermal nucleic acid sequence based amplification; self-sustained sequence replication assays;

boomerang DNA amplification; strand displacement activation; cycling probe technology; or any combination or variation thereof.

In preferred embodiments of the inventive methods, detecting an amplification product of the mammalian RNA or cDNA derived therefrom or signal derived therefrom is accomplished using a detection method such as gel electrophoresis; capillary electrophoresis; conventional enzyme-linked immunosorbent assay (ELISA) or modifications thereof, such as amplification using biotinylated or otherwise modified primers; nucleic acid hybridization using specific, detectably-labeled probes, such as fluorescent-, radioisotope-, or chromogenically-labeled probe; laser-induced fluorescence detection; Northern blot analysis; Southern blot analysis; electrochemiluminescence; reverse dot blot detection; and high-performance liquid chromatography.

In particularly preferred embodiments of the inventive methods, mammalian RNA is converted to cDNA using reverse transcriptase following extraction of RNA from a bodily fluid and prior to amplification.

The methods of the invention are advantageously used for providing a diagnosis or prognosis of, or as a predictive indicator for a non-neoplastic disease or pathologic condition or injury. The methods of the invention are particularly useful for providing a diagnosis or prognosis of, or monitoring of, or for providing a predictive indicator for, diseases or pathologic conditions of the heart and cardiovascular system. Cardiovascular disease is one of the most common potentially life-threatening non-neoplastic human diseases throughout the world. The methods of the invention enable diagnosis, detection, evaluation, and monitoring of cardiovascular disease, including but not limited to diseases and pathologic conditions of the heart such as myocardial infarction, myocardial

ischemia, coronary insufficiency, congestive heart failure, cardiomyopathy, atherosclerosis, intimal hyperplasia, and cardiac transplant rejection, and conditions associated with angina, and conditions and diseases associated with atherosclerosis and intimal hyperplasia or smooth muscle cell hyperplasia, and pathologic conditions and diseases associated with hypertension. The methods of the invention provide qualitative or quantitative detection of extracellular RNA in the blood plasma, serum, or other bodily fluid of a human, and wherein the RNA is associated with cardiovascular disease or pathologic conditions, including those of the heart and those of the vasculature, or with cells and tissues of the heart, arteries, and veins. Extracellular RNA associated with cardiovascular disease and pathologic conditions and/or injury includes, but is not limited to cardiac troponin T RNA (cTnT RNA), cardiac troponin I RNA (cTnI RNA), beta-myosin heavy chain RNA, acidic fibroblast growth factor RNA (heparin binding growth factor-1), basic fibroblast growth factor RNA, and platelet-derived growth factor-A and B RNA (PDGF-A RNA and PDGF-B RNA). It is to be understood that these RNA species provide examples and not limitation of the invention.

The methods of the invention are further particularly useful for providing a diagnosis or prognosis of, or for providing a predictive indicator for, diseases or pathologic conditions. The methods of the invention are applicable to non-neoplastic diseases and pathologic conditions affecting other organ systems, such as the nervous system. The methods of the invention enables diagnosing, detecting, evaluating, and monitoring of diseases and conditions of the central nervous system, including but not limited to stroke, ischemic brain injury, hypoxic conditions of the brain, head trauma, multiple sclerosis, Alzheimer's disease, encephalopathies, and neurodegenerative diseases. The inventive methods provide

qualitative or quantitative detection of extracellular mammalian RNA in the cerebrospinal fluid or other bodily fluid of an animal, most preferably a human, wherein the RNA is associated with a neurologic disease or condition such as injury or trauma, or with cells and tissues of the central nervous system. Extracellular RNA associated with neurologic disease and/or neurologic injury includes, but is not limited to the mutated presenilin 1 gene (PS1) RNA, mutated presenilin 2 gene (PS2) RNA, and Par 4 (prostate apoptosis response – 4) RNA. It is to be understood that these RNA species provide examples and not limitation of the invention.

The methods of the invention are also directed to non-neoplastic diseases and pathologic conditions affecting other solid organs and organ systems, such as those of the gastrointestinal system, the genitourinary system, the endocrine system, the respiratory system, the musculoskeletal system, and the skin. In these applications the method provides qualitative or quantitative detection of extracellular mammalian RNA in the blood plasma, serum, or other bodily fluid of an animal, most preferably a human, wherein the RNA is associated with a disease or pathologic condition of said organ or organ system and/or its cells and tissues. For example, cardiac troponin T mRNA (cTnT mRNA) is further detectable is some cases of skeletal muscle disease or pathologies such as Duchenne muscular dystrophy, polymyositis, and myopathy induced from end-stage renal disease.

In certain preferred embodiments of the methods of the invention, mammalian RNA associated with non-neoplastic, non-hematopoietic or non-hematological cells or tissue, or cDNA derived therefrom, is amplified in a quantitative manner, thereby enabling the quantitative comparison of said RNA or

cDNA present in a bodily fluid such as blood plasma, serum, or cerebrospinal fluid from a non-pregnant animal, preferably a human. In these embodiments, the amount of said RNA detected in bodily fluid from a particular individual animal is compared with a range of amounts of said RNA detected in said bodily fluid in healthy populations of animals, wherein increased amounts of RNA in said bodily fluid from the particular individual animal in comparison to healthy animals is indicative of a disease or pathologic condition, or is a predictive indicator of a disease or pathologic condition. In particularly preferred embodiments the non-neoplastic, non-hematological cells or tissue are terminally differentiated cells or tissue, or non-proliferative cells or tissue. In particularly preferred embodiments the cells or tissue are those of the heart, brain or muscle.

The methods of the invention further provide ways to identify animals, most preferably humans, having non-neoplastic disease or pathologic conditions, thereby permitting rational, informed treatment options to be used for making therapeutic decisions.

Another advantageous use for the methods of the invention is to provide a marker for assessing the adequacy of therapy, or for determining whether additional or more advanced or efficacious therapy is required. The invention therefore provides methods for developing a prognosis in such patients.

Another advantageous use for the methods of the invention is to provide for the screening of individuals as to determine their predisposition for a disease or pathologic condition, and further to determine their need for further diagnostic evaluation and/or for preventive therapy.

In a particularly preferred embodiment, the present invention provides methods for detecting extracellular cardiac troponin T mRNA or cardiac troponin I

mRNA and their isoforms in blood or blood fractions, including plasma and serum, or in other bodily fluid, in an animal, most preferably a human. As provided herein, the methods comprise the steps of extracting RNA from blood, blood plasma, serum, or other bodily fluid, *in vitro* amplifying cardiac troponin T mRNA or cDNA derived therefrom, and/or *in vitro* amplifying cardiac troponin I mRNA or cDNA derived therefrom, either qualitatively or quantitatively, and detecting the amplified product of cardiac troponin T mRNA or cDNA and/or of cardiac troponin I mRNA or cDNA.

In a first aspect of this embodiment, the present invention provides methods for detecting cardiac troponin T mRNA and/or cardiac troponin I mRNA in blood or blood fractions, including plasma and serum, or other bodily fluid in a human as a method for detecting, diagnosing, monitoring, prognosticating, or providing a predictive indicator for a disease or pathologic condition of the heart such as clinical or subclinical myocardial infarction or ischemic heart disease or coronary insufficiency.

In a particularly preferred embodiment, the present invention provides a method for detecting extracellular beta-myosin heavy chain mRNA in blood or blood fractions, including plasma and serum, or in other bodily fluid, in an animal, most preferably a human, the method comprising the steps of extracting RNA from blood, blood plasma, serum, or other bodily fluid, *in vitro* amplifying beta-myosin heavy chain mRNA or cDNA derived therefrom, either qualitatively or quantitatively, and detecting the amplified product of beta-myosin heavy chain mRNA or cDNA.

In a first aspect of this embodiment, the present invention provides methods for detecting beta-myosin heavy chain mRNA in blood or blood fractions,

including plasma and serum, or other bodily fluid in a human as a method for detecting, diagnosing, monitoring, prognosticating, or providing a predictive indicator for a disease or pathologic condition of the heart such as those associated with myocardial injury.

In a particularly preferred embodiment, the present invention provides a method for detecting extracellular acidic fibroblast growth factor mRNA (heparin-binding growth factor-1 mRNA) and/or extracellular basic fibroblast growth factor mRNA in blood or blood fractions, including blood plasma and serum, or other bodily fluid, in an animal, most preferably a human, the method comprising the steps of extracting RNA from blood, blood plasma, serum, or other bodily fluid, *in vitro* amplifying acidic fibroblast growth factor mRNA or cDNA derived therefrom, and/or basic fibroblast growth factor mRNA or cDNA derived therefrom, either qualitatively or quantitatively, and detecting the amplified product of acidic fibroblast growth factor mRNA or cDNA and/or basic fibroblast growth factor mRNA or cDNA.

In a first aspect of this embodiment, the present invention provides methods for detecting acidic fibroblast growth factor mRNA or basic fibroblast growth factor mRNA in blood or blood fractions, including blood plasma and serum, or other bodily fluid in an animal, most preferably a human, as a method for detecting, diagnosing, monitoring, prognosticating, or providing a predictive indicator for a disease or pathologic condition of vascular smooth muscle, most preferably atherosclerosis and/or intimal hyperplasia.

In a particularly preferred embodiment, the present invention provides a method for detecting extracellular prostate apoptosis response – 4 (Par-4) mRNA in cerebrospinal fluid, blood or blood fractions including plasma and serum, or

other bodily fluid, in an animal, most preferably a human, the method comprising the steps of extracting RNA from cerebrospinal fluid, blood, plasma, serum, or other bodily fluid, *in vitro* amplifying Par-4 mRNA or cDNA derived therefrom, either qualitatively or quantitatively, and detecting the amplified product of Par-4 mRNA or cDNA.

In a first aspect of this embodiment, the present invention provides methods for detecting Par-4 mRNA in cerebrospinal fluid, blood or blood fractions including plasma and serum, or other bodily fluid in a human as a method for detecting, diagnosing, monitoring, prognosticating, or providing a predictive indicator for a disease or pathologic condition or injury of the brain. In a particularly advantageous use of the invention, the disease or pathologic condition or injury of the brain is stroke, ischemia of the brain, hypoxia of the brain, traumatic brain injury, and/or neurodegenerative diseases.

The invention also provides diagnostic kits for use in the practice of the methods of the invention, specifically for the detection, diagnosis, monitoring, prognosticating, or predicting of non-neoplastic disease or pathologic disease or injury, wherein the diagnostic kit provides reagents for the extraction of mammalian RNA from plasma, serum, or other bodily fluid, and primers or probes used in the detection of the extracted RNA or cDNA derived therefrom.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides methods for diagnosing, evaluating, predicting within, or monitoring an animal, most preferably a human, for non-neoplastic diseases or pathologic conditions or injury by detecting extracellular mammalian RNA associated with said disease or pathologic condition or injury, such as but not limited to RNA derived from non-neoplastic, non-hematopoietic or non-hematological cells or tissue; RNA from terminally differentiated cells or tissue; RNA from non-proliferative cells, and/or RNA specific to cells or tissues of an organ(s) or organ system(s), wherein the RNA is detected in a bodily fluid of said animal, preferably blood and most preferably blood plasma and serum as well as in other bodily fluids, preferably cerebrospinal fluid, urine, saliva, effusions including pleural effusion, pericardial effusion, and joint effusion, ascites, cervical secretions, vaginal secretions, endometrial secretions, gastrointestinal secretions, sputum and bronchial secretions, and fluids associated with tissue lavages.

The invention further provides a method for detecting and/or monitoring mammalian RNA in blood plasma, serum, and/or bodily fluid from an animal, most preferably a human, or cDNA derived therefrom, that is associated with non-hematopoietic or non-hematological cells or tissue affected by a non-neoplastic disease or injury, wherein the method comprises the steps of extracting RNA from blood plasma, serum, or other bodily fluid, *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA derived therefrom, either qualitatively or quantitatively, and detecting the amplified product or signal of the RNA or cDNA derived therefrom.

The invention provides for the detection of mammalian RNA that does not contain viral or retroviral nucleic acid sequences within its own sequence. The

invention further provides for the detection of mammalian RNA that is not transcribed from a fragile site of genomic DNA, wherein a fragile site is a locus that is a frequent site of DNA strand breakage. Thus the invention further provides for detection of RNA that is transcribed from wild-type genomic DNA, in addition to detection of RNA transcribed from a mutated, deleted, translocated, methylated, or otherwise altered genomic DNA. The invention allows for the detection of messenger RNA, in addition to non-messenger RNA species such as ribosomal RNA, transfer RNA, ribonucleoprotein, and RNA transcribed from non-nuclear DNA.

The invention further provides a method for detecting and/or monitoring mammalian RNA in blood plasma, serum, and/or other bodily fluid from an animal, most preferably a human, or cDNA derived therefrom, that is associated with non-neoplastic, non-hematopoietic or non-hematological terminally differentiated cells or tissues or non-proliferative cells or tissues, including either healthy or diseased tissues, the method comprising the steps of extracting RNA from blood plasma, serum, or other bodily fluid, *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA derived therefrom either qualitatively or quantitatively, and detecting the amplified product or signal of RNA or cDNA derived therefrom, wherein the amplified product or signal is produced from an mRNA that is specific for non-neoplastic, non-hematopoietic or non-hematological terminally differentiated cells or tissues or non-proliferative cells or tissues.

The invention further provides methods for detecting and/or monitoring mammalian RNA associated with non-neoplastic, non-hematopoietic or non-hematological cells and tissues that are characteristic of specific tissue(s) and/or

organ(s) and/or organ system(s), either diseased or healthy, the methods comprising the steps of extracting RNA from blood plasma, serum, or other bodily fluid, *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA derived therefrom either qualitatively or quantitatively, and detecting the amplified product or signal of RNA or cDNA derived therefrom, wherein the amplified product or signal is produced from an mRNA that is specific for non-neoplastic, non-hematopoietic or non-hematological terminally differentiated cells or tissues or non-proliferative cells or tissues that are characteristic of specific tissue(s) and/or organ(s) and/or organ system(s), either diseased or healthy.

The invention further provides methods for detecting and/or monitoring mammalian RNA in blood, blood plasma, serum, and/or other bodily fluid from an animal, most preferably a human, or cDNA derived therefrom, that is derived from non-neoplastic cells or tissues, when said RNA produces a protein that has a consequent deleterious effect upon other differing non-neoplastic cells or tissues, thereby resulting in a disease or pathologic condition of the cells or tissues or their organ(s) and organ system(s) deleteriously affected, wherein the method comprises the steps of extracting RNA from blood plasma, serum, or other bodily fluid, *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA derived therefrom, either qualitatively or quantitatively, and detecting the amplified product or signal of the RNA or cDNA derived therefrom. For example, the methods of the invention may be used to detect within a bodily fluid mRNA associated with production of lipoproteins, wherein said mRNA is derived from cells of the liver, and wherein said protein has a deleterious effect upon the cells of the vascular system such as the arteries, thereby resulting in atherosclerosis.

In preferred embodiments of the methods of the invention, extracellular mammalian RNA is extracted from a bodily fluid of an animal, most preferably a human. From this extracted RNA, mRNA associated with a non-neoplastic disease or pathologic condition or injury, or derived from specific non-neoplastic cells, tissues, or organs of the animal, is then amplified, either after conversion into cDNA or directly, using *in vitro* amplification methods in either a qualitative or quantitative manner, or by amplification of a signal associated with the mRNA or cDNA derived therefrom in a qualitative or quantitative manner. The amplified product is then detected in either a qualitative or quantitative manner.

In additional preferred embodiments of the methods of the invention, organ-specific or tissue-specific or tissue-identifiable extracellular mammalian RNA present in a bodily fluid, most preferably blood plasma and serum, that is derived from specific non-neoplastic non-hematopoietic or non-hematological cells or tissue of an animal, most preferably a human, or cDNA derived therefrom, is hybridized to a specific primer, probe, solid substrate, or bioelectrical interface, the method comprising the extraction of RNA from a bodily fluid, most preferably plasma or serum, and hybridizing the RNA or cDNA derived therefrom to a specific primer, probe, solid substrate, or bioelectrical interface that consists of oligonucleotide sequences complimentary to RNA from specific non-neoplastic non-hematological cells and/or tissue, or cDNA derived therefrom. The invention thereby provides for the products of the hybridization.

In the practice of the methods of the invention, extracellular mammalian RNA may be extracted from any bodily fluid, including but not limited to whole blood, plasma, serum, cerebrospinal fluid, urine, saliva, effusions including pleural effusion, pericardial effusion, and joint effusions, ascites, cervical secretions,

vaginal secretions, endometrial secretions, gastrointestinal secretions, sputum and bronchial secretions, and fluids associated with tissue lavages, using, *for example,* extraction methods described in co-owned U.S. Patent No. 6,329,179 B1, the entire disclosure of which is hereby incorporated by reference. In the practice of the methods of the invention, extracellular mammalian RNA may be extracted from the bodily fluid using methods such as, but not limited to, gelatin extraction method; silica, glass bead, or diatom extraction method; guanidinium thiocyanate acid-phenol based extraction methods; guanidinium thiocyanate acid based extraction methods; methods using centrifugation through cesium chloride or similar gradients; phenol-chloroform based extraction methods; and/or other available RNA extraction methods, as are known in the art for use in extraction of intracellular RNA, including commercially available RNA extraction methods, *for example,* by using or adapting or modifying the methods of Boom *et al.* (1990 *J. Clin. Microbiology* 28: 495-503); Cheung et al. (1994 *J. Clin. Microbiology* 32: 2593-2597); Boom et al. (1991 *J. Clin. Microbiology* 29: 1804-1811); Chomczynski and Sacchi (1987 *Analytical Biochem.* 162:156-159); Chomczynski, (1993 *Biotech.* 15: 532-537); Chomczynski and Mackey (1995 *Biotechniques* 19: 942-945); Chomczynski and Mackey (1995 *Analytical Biochem.* 225: 163-164); Chirgwin et al. (1979 *Biochem.* 18: 5294-5299); Fournie et al. (1986 *Analytical Biochem.* 158: 250-256); the entire disclosure of said references hereby incorporated herein by reference in their entirety, and further as described in co-owned U.S. Patent No. 6,329,179 B1, the entire disclosure of which is hereby incorporated herein by reference in its entirety. It is further to be understood that any RNA extraction method that has demonstrated suitability for the extraction of tumor-derived or tumor-associated RNA from plasma or serum or other bodily

fluid is hereby recognized as being suitable for the extraction of non-neoplastic mammalian RNA from bodily fluid.

In particularly preferred embodiments of the invention, the extraction method used for extraction of extracellular mammalian RNA is a commercially available extraction method suitable for extraction of intracellular RNA, *for example*, TRIzol ™ (Life Technologies); Trisolv ™ (BioTecx Laboratories); ISOGEN ™ (Nippon Gene); RNA Stat ™ (Tel-test); TRI Reagent ™ (Sigma); SV Total RNA Isolation System (Promega); RNeasy Mini Kit (Qiagen); Perfect RNA: Total RNA Isolation Kit (Five Prime-Three Prime Inc., Boulder, Colorado); or similar commercially available kit, wherein extraction of RNA may be performed according to manufacturer's directions, adapted to the bodily fluid.

In a preferred embodiment, RNA is extracted from a bodily fluid using a probe or probes that specifically hybridize to specific RNA species, such as but not limited to probes attached to solid substrates or probes attached to magnetic beads or particles, or probes wherein upon hybridization to a nucleic acid, an electrical gradient or magnetic gradient or density gradient can thereby enable extraction and/or separation of specific RNA species from the remainder of bodily fluid. Further, the RNA or cDNA derived therefrom may be hybridized to a solid substrate at a bio-electrical interface whereupon hybridization of a specific RNA, or cDNA derived therefrom, generates an electrical signal which may further be amplified and detected.

In a preferred embodiment, the bodily fluid is either blood plasma or serum. It is preferred, but not required, that blood be processed soon after drawing, and preferably within three hours, as to minimize any nucleic acid degradation in the sample. In a preferred embodiment, blood is first collected by

venipuncture and kept on ice until further processing. Preferably, within 30 minutes to one hour of drawing the blood, serum is separated by centrifugation, for example at 1100 x g for 10 minutes at 4 degrees C. When using plasma, the blood is not permitted to coagulate prior to separation of the cellular and acellular components. Serum or plasma can be frozen, for example at –70 degrees C, after separation from the cellular portion of blood until further assayed, whereupon freezing the specimen can be maintained for extended periods (for example, several years) prior to assaying. When using frozen blood plasma or serum or other bodily fluid, the frozen serum or plasma or bodily fluid is rapidly thawed, for example in a 37 degree C water bath, and RNA is extracted therefrom without delay using methods as described above.

Following the extraction of RNA from a bodily fluid of an animal, a fraction of which contains a mammalian RNA associated with a non-neoplastic disease or pathologic disease or injury, or a fraction of which contains a mammalian RNA derived from cells or tissues of an organ or organ system of said animal, including but not limited to RNA derived from non-proliferating cells and tissues, and/or RNA derived from terminally differentiated cells and tissues, the RNA or cDNA derived therefrom is preferably amplified *in vitro*. Applicable amplification assays include but are not limited to amplifications assays detailed in co-owned U.S. Patent Application Serial No. 09/155,152, as herein incorporated by reference, and include but are not limited to reverse transcriptase polymerase chain reaction (RT-PCR), ligase chain reaction, RNA and cDNA signal amplification methods including branched chain signal amplification, amplifiable RNA reporters, Q-beta replication, transcription-based amplification, boomerang DNA amplification, strand displacement activation, cycling probe technology, isothermal

nucleic acid sequence based amplification, other self sustained sequence replication assays, and other nucleic acid amplification assays as known in the art, and/or any variations or combinations thereof, performed in either qualitative or quantitative fashion. For example, the methods of the invention can utilize nucleic acid amplification methods as known in the art, such as but not limited to adapting those described by Edmands et al. (1994 *PCR Methods Applic.* 3: 317-319); Abravaya et al. (1995 *Nucleic Acids Res.* 23: 675-682); Urdea et al. (1993 *AIDS* 7 (suppl 2): S11-S14); and/or Kievits et al. (1991 *J. Virological Methods* 35: 273-286); the entire disclosure of said references hereby incorporated by reference in their entirety.

In preferred embodiments of the methods of the invention, mammalian RNA is converted into cDNA using reverse transcriptase prior to *in vitro* amplification using methods known in the art. For example, a sample, such as 10 microL extracted serum RNA is reverse transcribed in a 30 microL volume containing 200 Units of Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI), a reaction buffer supplied by the manufacturer, 1 mM dNTPs, 0.5 micrograms random hexamers, and 25 Units of RNAsin (Promega, Madison, WI). Reverse transcription is typically performed under an overlaid mineral oil layer to inhibit evaporation and incubated at room temperature for 10 minutes followed by incubation at 37 degrees C for one hour. Alternatively, other methods well known in the art can be used to reverse transcribe the mammalian RNA to cDNA.

In the preferred embodiment, amplification primers or probes are specific for amplifying the mammalian RNA or cDNA derived therefrom associated with a non-neoplastic disease or pathologic condition, and/or associated with a non-

neoplastic and/or terminally differentiated and/or non-proliferative tissue from an organ or organ system. In a preferred embodiment, amplification is performed by RT-PCR, wherein amplification primers are specific for amplifying the cDNA. It is to be recognized that the design of said primers or probes is based upon the nucleic acid sequence of the RNA or cDNA, as known in the art, using methods as known in the art, and further as detailed in co-owned U.S. Patent No. 6,329,179 B1, the disclosure of which is incorporated herein by reference in its entirety.

In preferred embodiments of the inventive methods, following amplification the amplification product of the RNA or cDNA, or the amplified signal product of the RNA or cDNA, is then detected in either a qualitative or quantitative fashion. In preferred embodiments of the inventive methods, detecting an amplification product of the mammalian RNA or cDNA derived therefrom, or signal derived therefrom, is accomplished using a detection method such as but not limited to gel electrophoresis; capillary electrophoresis; conventional enzyme-linked immunosorbent assay (ELISA) or modifications thereof, such as amplification using biotinylated or otherwise modified primers; nucleic acid hybridization using specific, detectably-labeled probes, such as fluorescent-, radioisotope-, or chromogenically-labeled probe; laser-induced fluorescence detection; Northern blot analysis; Southern blot analysis; electrochemiluminescence; reverse dot blot detection; and high-performance liquid chromatography, wherein the methods of detection are performed using methods known in the art.

In one example of a preferred embodiment of the invention, cardiac troponin T mRNA is detected in a bodily fluid, most preferably blood, blood plasma, or serum, or in other bodily fluid. Detection of cardiac troponin T mRNA

in a bodily fluid is advantageous for the detection, diagnosis, monitoring, prognosticating, or providing a predictive indicator of non-neoplastic diseases and pathologic conditions of the heart, most preferably myocardial infarction, subclinical myocardial infarction and injury, and/or coronary insufficiency, including that associated with angina and unstable angina. In a preferred embodiment, amplification is performed by RT-PCR, preferably by the method of Townsend et al. (1995 *J. Mol. Cell. Cardiol.* 27: 2223-2236), or Messner et al. (2000 *Am. J. Clin. Pathol.* 114: 544-549) or Ricchiuti and Apple (1999 *Clin. Chem.* 45: 2129-2135); incorporated herein by reference in their entirety. In a preferred embodiment, the method set forth by Messner et al. (2000 *Am. J. Clin. Pathol.* 114: 544-549) is used, wherein nested RT-PCR is performed, wherein the preferred oligonucleotide primer sequences used in the first RT-PCR amplification reactions are as follows:

Primer 1: 5' GTTCTGAGGGAGAGCAGA (Sense; SEQ ID No. 1)

Primer 2: 5' AAGTGGTTTCTAGACGAGGA (Antisense; SEQ ID No. 2)

And wherein the preferred oligonucleotide primer sequences used in the second RT-PCR amplification reactions are as follows:

Primer 3: 5' GACCATGTCTGACATAGAAG (Sense; SEQ ID No. 3)

Primer 4: 5' CCGTCTCGTAGATATTGAAC (Antisense; SEQ ID No. 4)

In one example of a preferred embodiment of the invention, cardiac troponin T mRNA is harvested from serum or plasma, for example from an approximately 1.5 mL aliquot of serum or plasma, and RNA extracted therefrom the Perfect RNA Total RNA Isolation Kit (Five Prime-Three Prime) according to manufacturer's directions. From this extracted RNA preparation, 10 microL are then reverse transcribed to cDNA as described above. Nested RT-PCR for the

cardiac troponin T cDNA is performed using the method of Messner et al. (2000 *Am. J. Clin. Pathol.* 114: 544-549) incorporated herein by reference in its entirety, wherein PCR is performed using *Taq* DNA Polymerase and the Incubation Mix (with 1.5 mmol/L $MgCl_2$) from Appligene Oncor (Illkirch Cedex, France). Primers 1-4 as described above (SEQ ID Nos. 1-4) are utilized, with Primers 1 and 2 (SEQ ID Nos. 1 and 2) added to the mixture for the first stage of the PCR reaction, and Primers 3 and 4 (SEQ ID Nos. 3 and 4) added to the mixture for the second stage of the PCR reaction, for example using 10 picomoles each of Primer 1 and 2 (SEQ ID Nos. 1 and 2), and 10 picomoles each of Primer 3 and 4 (SEQ ID Nos. 3 and 4). The appropriate mixtures for each stage reaction are amplified in a thermocycler under a temperature profile consisting of 30 cycles of denaturation at 94 degrees C for 30 seconds, annealing at 60 degrees C for 30 seconds, and extension at 72 degrees C for 1 minute. Detection of the amplified product is then achieved, for example, by gel electrophoresis through a 1.5% agarose gel (Molecular Biology Grade Agarose, Gibco BRL), using ethidium bromide staining for visualization and identification of the product fragment, wherein the expected length for the cTnT3 isoform is 733 base pairs, and the expected length for the cTnT4 isoform is 634 base pairs.

The invention also provides alternative methods of amplification of cardiac troponin T mRNA or cDNA known in the art, including but not limited to the methods of Ricchiuti and Apple (1999 *Clin. Chem.* 45: 2129-2135), the disclosure of which is incorporated herein by reference in its entirety, and of Townsend et al. (1995 *J. Mol. Cell. Cardiol* 27: 2223-2236), the disclosure of which is incorporated herein by reference in its entirety.

The invention further provides for the cloning of the amplified product fragments into recombinant DNA replication vectors using standard techniques, for example for the cloning of cTnT mRNA or cDNA amplified products into pGEM-T vectors as described by Townsend et al (1995 *J. Mol. Cell. Cardiol.* 27: 2223-2236), the disclosure of which is incorporated herein by reference in its entirety. RNA can be produced from cloned PCR products, and in some instances the RNA expressed thereby, for example by using the Quick Coupled Transciption/Translation kit (Promega, Madison, WI) as directed by the manufacturer.

The invention further provides restriction enzyme digestion of an amplified product, such as the restriction enzyme digestion of a cTnT mRNA or cDNA amplified product and/or cTnI mRNA or cDNA amplified product, such as using the restriction enzymes *Hin*fI and *Msp*I (New England BioLabs, Beverly, MA), as described by Messner et al. (2000 *Clin. Chem.* 114: 544-549), the disclosure of which is incorporated by reference herein in its entirety. It will further be recognized that amplified products can be restriction enzyme digested prior to a second stage of amplification. Amplification methods can also be performed using primers specific for an internal control sequence, such as glyceraldehyde-3-phosphate dehydrogenase or c-abl, using methods as known to the art.

In another example of a preferred embodiment of the invention, cardiac troponin I mRNA (cTnI mRNA) is detected in a bodily fluid, most preferably blood, blood plasma, or serum, or in other bodily fluid. Detection of cardiac troponin I mRNA in a bodily fluid is advantageous for the detection, diagnosis, monitoring, prognosticating, or providing a predictive indicator of non-neoplastic diseases and pathologic conditions of the heart, most preferably myocardial

infarction, subclinical myocardial infarction or injury, and/or coronary insufficiency, including that associated with angina and unstable angina. In a preferred embodiment, amplification is performed by RT-PCR, preferably by the method of Messner et al. (2000 *Am. J. Clin. Pathol.* 114: 544-549), the disclosure of which is incorporated herein by reference in its entirety, or Ricchiuti and Apple (1999 *Clin. Chem.* 45: 2129-2135), the disclosure of which is incorporated herein by reference in its entirety. In a preferred embodiment, the method of Messner et al. (2000 *Am. J. Clin. Pathol.* 114: 544-549) is used, wherein nested RT-PCR is performed, wherein the preferred oligonucleotide primer sequences used in the first RT-PCR amplification reactions are as follows:

Primer 1: 5' AACCTCGCCCTGCACCAG (Sense; SEQ ID No. 5)

Primer 2: 5' CCCGGGACTCCTTATTTCG (Antisense; SEQ ID No. 6)

And wherein the preferred oligonucleotide primer sequences used in the second RT-PCR amplification reactions are as follows:

Primer 3: 5' CCTCCAACTACCGCGCTTA (Sense; SEQ ID No. 7)

Primer 4: 5' GACTCGGAAGGACGGATGA (Antisense; SEQ ID No. 8)

In one example of a preferred embodiment of the invention, cardiac troponin I mRNA is harvested from serum or plasma, for example from an approximately 1.5 mL aliquot of serum or plasma, and RNA extracted therefrom the Perfect RNA Total RNA Isolation Kit (Five Prime-Three Prime) according to the manufacturer's directions. From this extracted RNA preparation, 10 microL are then reverse transcribed to cDNA as described above. Nested RT-PCR for the cardiac troponin I cDNA is performed using the method of Messner et al. (2000 *Am. J. Clin. Pathol.* 114: 544-549) incorporated herein by reference in its entirety, wherein PCR is performed using *Taq* DNA Polymerase and the Incubation Mix

(with 1.5 mmol/L $MgCl_2$) from Appligene Oncor (Illkirch Cedex, France). Primers 1-4 as described above (SEQ ID Nos. 5-8) are utilized, with Primers 1 and 2 (SEQ ID Nos. 5 and 6) added to the mixture for the first stage of the PCR reaction, and Primers 3 and 4 (SEQ ID Nos. 7 and 8) added to the mixture for the second stage of the PCR reaction, for example using 10 picomoles each of Primer 1 and 2 (SEQ ID Nos. 5 and 6), and 10 picomoles each of Primer 3 and 4 (SEQ ID Nos. 7 and 8). The appropriate mixtures for each stage reaction are amplified in a thermocycler under a temperature profile consisting of 30 cycles of denaturation at 94 degrees C for 30 seconds, annealing at 60 degrees C for 30 seconds, and extension at 72 degrees C for 1 minute. Detection of the amplified product is then achieved, for example, by gel electrophoresis through a 1.5% agarose gel (Molecular Biology Grade Agarose, Gibco BRL), using ethidium bromide staining for visualization and identification of the product fragment, wherein the expected length for the cTnI amplification product is 581 base pairs.

The invention provides for alternative methods of amplification of cardiac troponin I mRNA or cDNA known in the art, including but not limited to the methods of Ricchiuti and Apple (1999 *Clin. Chem.* 45: 2129-2135), the disclosure of which is incorporated herein by reference in its entirety.

In another example of a preferred embodiment of the invention, beta-myosin heavy chain mRNA is detected in a bodily fluid of an animal, most preferably in blood, blood plasma, or serum or in other bodily fluid. Detection of beta-myosin heavy chain mRNA in a bodily fluid is advantageous for the detection, diagnosis, monitoring, prognosticating, or providing a predictive indicator of non-neoplastic diseases and pathologic conditions of muscle, most advantageously cardiac muscle of the heart. In one example of a preferred

embodiment of the invention, beta-myosin heavy chain mRNA is harvested serum or plasma, for example from an approximately 1.5 mL aliquot of serum or plasma, and RNA extracted therefrom using the Perfect RNA Total RNA Isolation Kit (Five Prime- Three Prime) according to the manufacturer's directions. From this extracted RNA preparation, 10 microL are then reverse transcribed to cDNA as described above. The cDNA is then hybridized to a primer or probe specific to beta-myosin heavy chain cDNA, most preferably an oligonucleotide primer or probe, wherein the primer or probe is specific to the nucleotide sequence of a fragment of beta-myosin heavy chain cDNA. Alternatively, extracted mRNA may be hybridized directly to a probe specific to the nucleotide sequence of a fragment of the mRNA. Hybridized primers or probes may thereby enable either qualitative or quantitative amplification or signal amplification of the mRNA or cDNA derived therefrom, such as beta-myosin heavy chain cDNA, followed by detection of the product, by methods of the art as previously described.

In another example of a preferred embodiment of the invention, acidic fibroblast growth factor mRNA and/or basic fibroblast growth factor mRNA is detected in a bodily fluid, most preferably blood, blood plasma and serum, or other bodily fluid. Detection of acidic fibroblast growth factor mRNA and/or basic fibroblast growth factor mRNA in a bodily fluid is advantageous for the detection, diagnosis, monitoring, prognosticating, or providing a predictive indicator of non-neoplastic diseases and pathologic conditions of the cardiovascular system, most preferably non-neoplastic diseases and pathologic conditions relating to atherosclerosis and intimal hyperplasia. In a preferred embodiment, amplification is performed by RT-PCR, preferably by the method of Zhao et al. (1994

Circulation 90: 677-685) but preferably for 45 cycles, the disclosure of which is incorporated herein by reference in its entirety.

In one example of a preferred embodiment of the invention, acidic fibroblast growth factor mRNA and/or basic fibroblast growth factor mRNA is harvested from blood, most preferably blood plasma or serum, or other bodily fluid, for example from an approximately 1.5 mL aliquot of serum or plasma, and RNA extracted therefrom using the Perfect RNA Total RNA Isolation Kit (Five Prime- Three Prime) according to the manufacturer's directions. From this extracted RNA preparation, 10 microL are then reverse transcribed to cDNA as described above. RT-PCR for acidic fibroblast growth factor cDNA and/or basic fibroblast growth factor cDNA is performed using the method of Zhao et al. (1994 *Circulation* 90:677-685) but preferably for 45 cycles, the disclosure of which is incorporated herein by reference in its entirety, with the amplified product detected as previously described, for example by gel electrophoresis with ethidium bromide staining.

In another example of a preferred embodiment of the invention, prostate apoptosis response-4 (Par-4) mRNA is detected in a bodily fluid, most preferably cerebrospinal fluid, or blood, blood plasma, serum, or other bodily fluid. Detection of Par-4 mRNA in a bodily fluid is advantageous for the detection, diagnosis, monitoring, prognosticating, or providing a predictive indicator of non-neoplastic diseases and pathologic conditions and injuries of the brain and nervous system, such as stroke, ischemia of the brain, hypoxia of the brain, traumatic brain injury, and neurodegenerative disease. In a preferred embodiment, amplification is performed by RT-PCR, preferably by the method of Dhillon et al. (2001 *Exp.*

Neurol. 170: 140-148) but preferably for 45 cycles, the disclosure of which is incorporated herein by reference in its entirety.

In one example of a preferred embodiment of the invention, Par-4 mRNA is harvested from cerebrospinal fluid or serum or plasma, for example from an aliquot of cerebrospinal fluid, and RNA extracted therefrom using the Perfect RNA Total RNA Isolation Kit (Five Prime-Three Prime) according to manufacturer's directions. From this extracted RNA preparation, 10 microL are then reverse transcribed to cDNA as described above. RT-PCR for Par-4 cDNA is performed using the method of Dhillon et al. (2001 *Exp. Neurol.* 170: 140-148) but preferably for 45 cycles, the disclosure of which is incorporated herein by reference in its entirety, with the amplified product detected as previously described, for example by gel electrophoresis with ethidium bromide staining.

In particularly advantageous methods of the invention, a multiplexed panel or sequential analysis or cDNA chip approach is employed to allow the concurrent or sequential analysis of multiple RNA from a bodily fluid specimen. In one aspect of this embodiment, multiple mammalian RNA associated with a particular organ or organ system are thereby detected in a bodily fluid, most preferably blood, blood plasma, serum, or other bodily fluid, as a method for detecting, diagnosing, monitoring, predicting, or prognosticating a non-neoplastic disease or pathologic condition or injury. In a particularly advantageous embodiment of the invention, cardiac troponin T mRNA and cardiac troponin I mRNA or other myocardial-derived RNA such as beta-myosin heavy chain mRNA are detected in sequential, concurrent, multiplexed, or chip fashion from the same bodily fluid specimen, most preferably blood, blood plasma, serum, or other bodily fluid.

In an embodiment of the invention, the RNA of interest is compared to RNA from a housekeeper gene or genes similarly extracted from the bodily fluid in either quantitative or qualitative fashion.

In another embodiment of the invention, mammalian RNA from a bodily fluid specimen of an animal, most preferably a human, is concurrently or sequentially analyzed in comparison with protein markers or lipoprotein markers or DNA markers from said bodily fluid specimen in qualitative or quantitative fashion, wherein comparative analysis of the presence of mammalian RNA in said bodily fluid specimen to the presence of the protein or DNA in said bodily specimen facilitates the diagnosis, detection, evaluation, monitoring, prognosticating, or predicting of a non-neoplastic disease or pathologic condition or injury in said animal. For example, mammalian RNA such as but not limited to acidic fibroblast growth factor mRNA and/or basic fibroblast growth factor mRNA and/or platelet-derived growth factor mRNA may be detected in blood and sequentially or concurrently compared with serum lipoproteins and/or serum cholesterol as a method of prognosticating or predicting atherosclerotic disease.

The examples of preferred embodiments of the invention provided herein whereby cardiac troponin T mRNA or cardiac troponin I mRNA or beta-myosin heavy chain mRNA or acidic fibroblast growth factor mRNA or basic fibroblast growth factor mRNA or Par-4 mRNA are detected in bodily fluid are provided as examples and not as limitations on the methods of the invention. It is to be understood that the invention generally encompasses detection of extracellular mammalian RNA associated with non-neoplastic disease or pathologic condition or injury in an animal, and/or RNA derived from non-neoplastic terminally differentiated or non-proliferative tissue of an animal, and/or RNA derived from

non-neoplastic organ-specific tissue of an animal, wherein the RNA is detected in a bodily fluid taken from said animal. It will be understood in the art that other RNA may provide markers of pathologic conditions or disease or injury, and it is within the scope and spirit of the invention that these RNA may be extracted as extracellular RNA from blood plasma, serum, or other bodily fluid, the RNA species of interest or cDNA derived therefrom can be amplified or signal amplified using primers or probes specific to the RNA or cDNA of interest, and the amplified product or signal be detected, as is taught by the invention herein.

In a particularly preferred embodiment, the mammalian RNA associated with a non-neoplastic disease or pathologic condition or cDNA derived therefrom is amplified or signal amplified in a quantitative amplification reaction. Quantitative amplification of the mammalian RNA or cDNA is particularly advantageous when said RNA is present at lower levels in a bodily fluid of healthy animals, but present at higher levels in a bodily fluid of animals with a disease or pathologic condition or injury. The method thereby enables statistically-based discrimination between individuals with a disease or pathologic condition and healthy populations or populations without the disease or pathologic condition. The quantitative method further enables comparison between individuals having the disease or condition, wherein higher levels of said RNA in a bodily fluid is indicative of a disease or pathologic condition of greater severity, or of earlier onset. The quantitative method thereby provides a method for monitoring a disease or pathologic condition, or monitoring a response to therapy for a disease or pathologic condition, or for determining a prognosis. The methods of the invention thereby provide a marker for assessing the adequacy of therapy, or for determining whether additional or more advanced therapy is required. It is

particularly advantageous to perform the methods of the invention in a serial manner to monitor an animal's disease or condition, and to assess the adequacy of therapy or the need to change therapy. The methods of the invention thereby further permit rational, informed treatment options to be used for making therapeutic decisions.

The methods of the invention are thereby advantageously used for providing a diagnosis or prognosis of, or as a predictive indicator for a non-neoplastic disease or pathologic condition or injury. The methods of the invention are particularly useful for providing a diagnosis or prognosis of, or monitoring of, or for providing a predictive indicator for cardiovascular diseases and conditions. Thus, the methods of the invention will be useful in the assessment of individuals having symptoms that might be consequent to a cardiovascular disease or condition. The methods of the invention will further be useful in the assessment of individuals having risk factors for a cardiovascular disease or condition. The methods of the invention will further be useful for the monitoring or determining prognosis of individuals known to have a cardiovascular disease or condition. The methods of the invention will thus be useful either alone or in conjunction with other tests, assays, procedures, or exams that enable the evaluation of cardiovascular diseases and conditions, such as but not limited to stress tests, radiologic scans, echocardiogram, and electrocardiograms. The methods of the invention will further be useful to monitor an individual during or following surgery.

The methods of the invention are further particularly useful for providing a diagnosis or prognosis of, or as a predictive indicator for a non-neoplastic neurologic disease or neurologic pathologic condition or injury. Thus, the methods

of the invention will be useful in the assessment of individuals having symptoms that might be consequent to a neurologic disease or condition. The methods of the invention will further be useful in the assessment of individuals having risk factors for a neurologic disease or condition. The methods of the invention will further be useful for the monitoring or determining prognosis of individuals known to have a neurologic disease or condition. The methods of the invention will thus be useful either alone or in conjunction with other tests, assays, procedures, or exams that enable the evaluation of neurologic diseases and conditions, such as but not limited to radiologic exams such as CT scan and MRI scan, electroencephalogram, and lumbar puncture. The methods of the invention will further be useful to monitor an individual during or following surgery.

The methods of the invention will further be advantageous in the screening of individuals for predisposition to diseases and pathologic conditions, thereby enabling the institution of preventive therapy.

The methods of the invention provides for diagnostic kits for the detection, diagnosis, monitoring, prognosticating, or predicting of non-neoplastic disease or pathologic condition or injury, wherein the diagnostic kit provides for the extraction of mammalian RNA from plasma, serum, or other bodily fluid, and/or provides primers or probes used in the detection of the extracted RNA of interest or cDNA derived therefrom.

The methods of the invention and preferred uses for the methods of the invention are more fully illustrated in the following Example. This Example illustrates certain aspects of the above-described method and advantageous results. This Example is shown by way of illustration and not by way of limitation.

EXAMPLE 1

A 52 year-old man presents to his doctor with complaints of recent onset of increasingly frequent episodes of mild chest discomfort. His doctor suspects a possible cardiac etiology, and orders further cardiac evaluation. The man undergoes a "stress test" consisting of an electrocardiogram test during and following controlled treadmill exercise. Peripheral venous blood is drawn from the man one hour and six hours following the stress test to evaluate for the presence of cardiac troponin T mRNA and cardiac troponin I mRNA using the methods of the invention. Five ml of blood plasma is collected for each time period, maintained on ice until separation of plasma from the cellular blood fraction, and then frozen until further testing. Both plasma samples are evaluated in a laboratory at the same time by rapidly thawing the frozen samples, extracting RNA from the plasma using a commercial RNA extraction kit such as the Perfect RNA Total RNA Isolation Kit (Five Prime-Three Prime) according to manufacturer's directions, reverse transcribing the extracted RNA to cDNA as previously described, and amplifying the cDNA with primers specific for cardiac troponin T cDNA and cardiac troponin I cDNA by the methods of the invention, such as by using the method of Messner et al. (2000 *Am. J. Clin. Pathol.* 114: 544-549), performed in a qualitative fashion. The amplified product is then detected, such as by using gel electrophoresis. Detection of cardiac troponin T mRNA and/or cardiac troponin I mRNA in the peripheral blood would indicate an underlying cardiovascular disease associated with cellular injury during the stress test, and the doctor in this case would thereby make a diagnosis of unstable angina and would thereby institute therapeutic measures.

Five weeks following the treadmill the patient presents to the emergency room with complaints of sustained substernal chest discomfort and shortness of breath. The emergency doctor suspects a possible myocardial infarction. To confirm this, he obtains peripheral venous blood from the patient and evaluate the blood for the presence of cardiac troponin T mRNA and/or cardiac troponin I mRNA in the peripheral blood, using the methods of the invention described. The presence of cardiac troponin T mRNA and cardiac troponin I mRNA is thereby confirmed and a diagnosis of myocardial infarction thereby made, and the man is admitted to the hospital coronary care unit. There, cardiac troponin T mRNA and cardiac troponin I mRNA in blood would be serially quantitatively monitored using the method of the invention as a means of monitoring the progression of the myocardial infarction, the severity of the myocardial tissue injury, and the prognosis for the patient. In addition, plasma beta-myosin heavy chain mRNA is monitored using the method of the invention to further evaluate the severity of the myocardial tissue injury.

What is claimed:

1. A method for detecting, diagnosing, monitoring, or predicting a non-neoplastic disease of an organ in a human or animal, the method comprising the step of detecting extracellular mammalian RNA in a bodily fluid of a human or animal, wherein said RNA is present in non-hematological cells of the diseased organ, and not present in hematological cells of the human or animal.

2. A method for detecting, diagnosing, monitoring, or predicting a non-neoplastic non-virally mediated disease in a human or animal, the method comprising the step of detecting non-neoplastic non-viral extracellular mammalian RNA in a bodily fluid from said human or animal in a quantitative fashion, wherein said RNA is present in the bodily fluid of a human or animal with a non-viral non-neoplastic disease in quantitative amounts that are greater than present in the bodily fluid of a human or animal without disease.

3. A method for detecting non-viral mammalian extracellular RNA in blood, blood plasma or serum, or other bodily fluid from a human or animal without cancer, wherein said RNA is derived from non-hematological cells or tissue of the human or animal having a non-neoplastic disease or pathologic condition or injury of said tissue, the method comprising the steps of:

a) extracting RNA from blood, blood plasma, serum, or other bodily fluid;

b) *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA derived therefrom in a quantitative or qualitative fashion using primers or probes specific for the RNA or cDNA derived from non-hematological cells;

c) detecting the amplified product or signal produced thereby.

4. A method of detecting non-viral mammalian extracellular RNA in blood, blood plasma, serum, or other bodily fluid of a non-pregnant human or animal without cancer, wherein said RNA is not derived from hematological cells or from a fragile site, the method comprising the steps of:

a) extracting RNA from blood, blood plasma, serum, or other bodily fluid;

b) *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA derived therefrom in qualitative or quantitative fashion using primers or probes specific for non-viral mammalian RNA or cDNA derived therefrom, said RNA not derived from hematological cells or from a fragile site;

c) detecting the amplified product or signal produced thereby.

5. The method of claim 3 wherein the cells or tissue are heart cells or tissue.

6. The method of claim 3 wherein the cells or tissue are brain cells or tissue.

7. A method of detecting extracellular mammalian RNA in blood, blood plasma, serum, or other bodily fluid of a non-pregnant human or animal without cancer, wherein said RNA is derived from terminally differentiated non-hematopoietic cells or tissue of the human or animal, the method comprising the steps of:

   a) extracting RNA from blood, blood plasma, serum or other bodily fluid;

   b) *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA derived therefrom in a qualitative or quantitative fashion using primers or probes specific for RNA derived from terminally differentiated non-hematopoietic cells or tissue of the human or animal;

   c) detecting the amplified product or signal produced thereby.

8. A method of detecting extracellular mammalian RNA in blood, blood plasma, serum, or other bodily fluid of a non-pregnant human or animal without cancer, wherein said RNA is derived from non-proliferative non-hematopoietic cells or tissue of the human or animal, the method comprising the steps of:

   a) extracting RNA from blood, blood plasma, serum, or other bodily fluid;

   b) *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA derived therefrom in a qualitative or quantitative fashion using primers or probes specific for RNA derived from non-

proliferative non-hematopoietic cells or tissue of the human or animal;

c) detecting the amplified product or signal produced thereby.

9. A method of detecting mammalian RNA in blood plasma, serum, or other bodily fluid of a human or animal, wherein said RNA is derived from cells or tissues of the heart or arteries or veins of the human or animal, the method comprising the steps of:

a) extracting RNA from blood plasma, serum, or other bodily fluid;

b) *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA derived therefrom in a qualitative or quantitative fashion using primers or probes specific for RNA derived from cells or tissues of the heart or arteries or veins;

c) detecting the amplified product or signal produced thereby.

10. A method of detecting non-viral mammalian extracellular RNA in cerebrospinal fluid, blood plasma, serum, or other bodily fluid of a human or animal, wherein said RNA is derived from cells or tissues of the brain of the human or animal, the method comprising the steps of:

a) extracting RNA from cerebrospinal fluid, blood plasma, serum, or other bodily fluid;

b) *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA derived therefrom in a qualitative or quantitative fashion using primers or probes specific for non-viral mammalian RNA derived from cells or tissues of the brain;

c) detecting the amplified product or signal produced thereby.

11. The method of claim 7, wherein the cells or tissues are heart cells or tissue.

12. The method of claim 7, wherein the cells or tissue are brain cells or tissue.

13. A method according to claim 3, whereby a non-neoplastic disease or pathologic condition or injury is detected, diagnosed, monitored, prognosticated, or predicted.

14. A method according to claim 4, whereby a non-neoplastic disease or pathologic condition or injury is detected, diagnosed, monitored, prognosticated, or predicted.

15. A method according to claim 7, whereby a non-neoplastic disease or pathologic condition or injury is detected, diagnosed, monitored, prognosticated, or predicted.

16. A method according to claim 8, whereby a non-neoplastic disease or pathologic condition or injury is detected, diagnosed, monitored, prognosticated, or predicted.

17. The method of hybridizing non-viral mammalian RNA from a non-pregnant human or animal without cancer, said RNA being derived from a non-fragile genomic locus of a non-hematopoietic cell, to a primer, probe,

solid substrate, or bioelectrical interface, the method comprising the steps of:

a) extracting RNA from blood plasma or serum from a non-pregnant human or animal without cancer;

b) hybridizing a portion of the extracted RNA or cDNA derived therefrom to a primer, probe, or solid substrate, wherein the primer, probe, solid substrate, or bioelectrical interface consists of oligonucleotide sequences complementary to RNA or cDNA derived from a non-fragile genomic locus of a non-hematopoietic cell.

18. The hybridized product of claim 17.

19. A method according to claim 17, wherein the cell is a heart or vasculature cell.

20. A method according to claim 17, wherein the cell is a brain cell.

21. A method of detecting non-viral mammalian extracellular RNA in blood, blood plasma, serum, or other bodily fluid of a human or animal without cancer, wherein said RNA translates a protein that has a deleterious effect upon other cells or tissues within the animal, thereby resulting in a disease or pathologic condition in the human or animal, the method comprising the steps of:

a) extracting RNA from blood, blood plasma, serum or other bodily fluid from a human or animal without cancer;

b) *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA derived therefrom in a qualitative or quantitative fashion using primers or probes specific for a RNA that translates said protein having deleterious effect;

c) detecting the amplified product or signal produced thereby.

22. A method according to claim 21, whereby a non-neoplastic disease or pathologic condition or injury is detected, diagnosed, monitored, prognosticated, or predicted.

23. The method of claim 3, wherein the amplification in step (b) is performed by an RNA amplification method that amplifies RNA directly or wherein the RNA is first reverse transcribed to cDNA, whereby the cDNA is amplified, wherein the amplification method is reverse transcriptase polymerase chain reaction, ligase chain reaction, branched DNA signal amplification, amplifiable RNA reporters, Q-beta replication, transcription-based amplification, isothermal nucleic acid sequence based amplification, self-sustained sequence replication assays, boomerang DNA amplification, strand displacement activation, cycling probe technology, and/or modifications or variations thereof.

24. The method of claim 4, wherein the amplification in step (b) is performed by an RNA amplification method that amplifies the RNA directly or wherein the RNA is first reverse transcribed to cDNA, whereby the cDNA is amplified, wherein the amplification method is reverse transcriptase

polymerase chain reaction, ligase chain reaction, branched DNA signal amplification, amplifiable RNA reporters, Q-beta replication, transcription-based amplification, isothermal nucleic acid sequence based amplification, self-sustained sequence replication assays, boomerang DNA amplification, strand displacement activation, cycling probe technology, and/or modifications or variations thereof.

25. The method of claim 7, wherein the amplification in step (b) is performed by an RNA amplification method that amplifies RNA directly or wherein the RNA is reverse transcribed to cDNA, whereby the cDNA is amplified, wherein the amplification method is reverse transcriptase polymerase chain reaction, ligase chain reaction, branched DNA signal amplification, amplifiable RNA reporters, Q-beta replication, transcription-based amplification, isothermal nucleic acid sequence based amplification, self-sustained sequence replication assays, boomerang DNA amplification, strand displacement activation, cycling probe technology, and/or modifications or variations thereof.

26. The method of claim 8, wherein the amplification in step (b) is performed by an RNA amplification method that amplifies the RNA directly or wherein the RNA is first reverse transcribed to cDNA, whereby the cDNA is amplified, wherein the amplification method is reverse transcriptase polymerase chain reaction, ligase chain reaction, branched DNA signal amplification, amplifiable RNA reporters, Q-beta replication, transcription-based amplification, isothermal nucleic acid sequence based amplification,

self-sustained sequence replication assays, boomerang DNA amplification, strand displacement activation, cycling probe technology, and/or combinations or variations thereof.

27. The method of claim 21, wherein the amplification in step (b) is performed by an RNA amplification method that amplifies the RNA directly or wherein the RNA is first reverse transcribed to cDNA, whereby the cDNA is amplified, wherein the amplification method is reverse transcriptase polymerase chain reaction, ligase chain reaction, branched DNA signal amplification, amplifiable RNA reporters, Q-beta replication, transcription-based amplification, isothermal nucleic acid sequence based amplification, self-sustained sequence replication assays, boomerang DNA amplification, strand displacement activation, cycling probe technology, and/or combinations or variations thereof.

28. A method wherein non-viral mammalian RNA extracted from the blood plasma or serum of a non-pregnant human or animal, said RNA associated with a non-neoplastic disease and not derived from a fragile locus, is reverse transcribed to cDNA.

29. A method wherein non-viral mammalian RNA extracted from the blood plasma or serum of a non-pregnant human or animal, said RNA associated with a non-neoplastic disease and derived from non-hematological terminally differentiated cells of an organ, is reverse transcribed to cDNA.

30. A method wherein non-viral mammalian extracellular RNA extracted from the bodily fluid of a non-pregnant human or animal, said RNA associated with a non-neoplastic disease and not derived from a fragile locus, is reverse transcribed to cDNA.

31. A method wherein non-viral mammalian extracellular RNA extracted from a bodily fluid of a human or animal, said RNA associated with a non-neoplastic disease and derived from non-hematological terminally differentiated cells of an organ, is reverse transcribed to cDNA.

32. The method of claim 3, wherein detection of the amplified product in step c is performed using a detection method that is gel electrophoresis, capillary electrophoresis, ELISA detection using biotinylated or otherwise modified primers, labeled fluorescent or chromogenic probes, laser-induced fluorescence, Northern blot analysis, Southern blot analysis, electrochemiluminescense, reverse dot blot detection, or high-performance liquid chromatography.

33. The method of claim 4, wherein detection of the amplified product in step c is performed using a detection method that is gel electrophoresis, capillary electrophoresis, ELISA detection using biotinylated or otherwise modified primers, labeled fluorescent or chromogenic probes, laser-induced fluorescence, Northern blot analysis, Southern blot analysis, electrochemiluminescence, reverse dot blot detection, or high-performance liquid chromatography.

34. The method of claim 7, wherein detection of the amplified product in step c is performed using a detection method that is gel electrophoresis, capillary electrophoresis, ELISA detection using biotinylated or otherwise modified primers, labeled fluorescent or chromogenic probes, laser-induced fluorescence, Northern blot analysis, Southern blot analysis, electrochemiluminescence, reverse dot blot detection, or high-performance liquid chromatography.

35. The method of claim 8, wherein detection of the amplified product in step c is performed using a detection method that is gel electrophoresis, capillary electrophoresis, ELISA detection using biotinylated or otherwise modified primers, labeled fluorescent or chromogenic probes, laser-induced fluorescence, Northern blot analysis, Southern blot analysis, electrochemiluminescence, reverse dot blot detection, or high-performance liquid chromatography.

36. The method of claim 21, wherein detection of the amplified product in step c is performed using a detection method that is gel electrophoresis, capillary electrophoresis, ELISA detection using biotinylated or otherwise modified primers, labeled fluorescent or chromogenic probes, laser-induced fluorescence, Northern blot analysis, Southern blot analysis, electrochemiluminescence, reverse dot blot detection, or high-performance liquid chromatography.

37. A method according to claim 1, wherein the disease is a cardiovascular disease.

38. A method according to claim 2, wherein the disease is a cardiovascular disease.

39. A method according to claim 13, wherein the disease is a cardiovascular disease.

40. A method according to claim 14, wherein the disease is a cardiovascular disease.

41. A method according to claim 15, wherein the disease is a cardiovascular disease.

42. A method according to claim 16, wherein the disease is a cardiovascular disease.

43. A method according to claim 1, wherein the disease is a neurologic disease.

44. A method according to claim 2, wherein the disease is a neurologic disease.

45. A method according to claim 13, wherein the disease is a neurologic disease.

46. A method according to claim 14, wherein the disease is a neurologic disease.

47. A method according to claim 15, wherein the disease is a neurologic disease.

48. A method according to claim 16, wherein the disease is a neurologic disease.

49. A method according to claim 3, further comprising the step of initiating or maintaining a therapy in a human or animal when the amplified product or signal is detected.

50. A method according to claim 4, further comprising the step of initiating or maintaining a therapy in a human or animal when the amplified product or signal is detected.

51. A method according to claim 21, further comprising the step of initiating or maintaining a therapy in a human or animal when the amplified product or signal is detected.

52. A method of detecting cardiac troponin T mRNA in blood, blood plasma, serum, or other bodily fluid from a human or animal, the method comprising the steps of:

a) extracting RNA from blood, blood plasma, serum, or other bodily fluid from a human or animal;

b) *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA prepared therefrom, wherein said fraction comprises cardiac troponin T mRNA, and wherein amplification is performed in either a qualitative or quantitative fashion;

c) detecting the amplified cardiac troponin T mRNA or cDNA product or signal.

53. A method according to claim 52, wherein a non-neoplastic cardiovascular disease, condition, or injury is detected, diagnosed, monitored, prognosticated, or predicted.

54. A method of detecting cardiac troponin I mRNA in blood, blood plasma, serum, or other bodily fluid from a human or animal, the method comprising the steps of:

a) extracting RNA from blood, blood plasma, serum, or other bodily fluid from a human or animal;

b) *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA prepared therefrom, wherein said fraction comprises cardiac troponin I mRNA, and wherein amplification is performed in either a qualitative or quantitative fashion;

c) Detecting the amplified cardiac troponin I mRNA or cDNA product or signal.

55. A method according to claim 54, wherein a non-neoplastic cardiovascular disease, condition, or injury is detected, diagnosed, monitored, prognosticated, or predicted.

56. A method of detecting beta-myosin heavy chain mRNA in blood, blood plasma, serum, or other bodily fluid from a human or animal, the method comprising the steps of:

a) extracting RNA from blood, blood plasma, serum, or other bodily fluid from a human or animal;

b) *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA prepared therefrom, wherein said fraction comprises beta-myosin heavy chain mRNA, and wherein amplification is performed in either a qualitative or quantitative fashion

c) detecting the amplified beta-myosin heavy chain mRNA or cDNA product or signal.

57. A method according to claim 56, wherein a non-neoplastic cardiovascular disease, condition, or injury is detected, diagnosed, monitored, prognosticated, or predicted.

58. A method of detecting acidic fibroblast growth factor mRNA in plasma and/or serum from a human or animal, the method comprising the steps of:

a) extracting RNA from plasma or serum from a human or animal;

b) *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA prepared therefrom, wherein said fraction comprises

acidic fibroblast growth factor mRNA, and wherein amplification is performed in either a qualitative or quantitative fashion;

c) detecting the amplified acidic fibroblast growth factor mRNA or cDNA product or signal.

59. A method according to claim 58, wherein a non-neoplastic cardiovascular disease, condition, or injury is detected, diagnosed, monitored, prognosticated, or predicted.

60. A method of detecting basic fibroblast growth factor mRNA in plasma or serum of a human or animal, the method comprising the steps of:

a) extracting RNA from plasma or serum from a human or animal;

b) *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA prepared therefrom, wherein said fraction comprises basic fibroblast growth factor mRNA, and wherein amplification is performed in either a qualitative or quantitative fashion;

c) detecting the amplified basic fibroblast growth factor mRNA or cDNA product or signal.

61. A method according to claim 60, wherein a non-neoplastic cardiovascular disease, condition, or injury is detected, diagnosed, monitored, prognosticated, or predicted.

62. A method of detecting Par-4 mRNA in cerebrospinal fluid, blood, blood plasma, serum, or other bodily fluid from a human or animal, the method comprising the steps of:

a) extracting RNA from cerebrospinal fluid, blood, blood plasma, serum, or other bodily fluid from a human or animal;

b) *in vitro* amplifying or signal amplifying a fraction of the extracted RNA or cDNA prepared therefrom, wherein said fraction comprises Par-4 mRNA, and wherein amplification is performed in either a qualitative or quantitative fashion;

c) detecting the amplified Par-4 mRNA or cDNA product or signal.

63. A method according to claim 62, wherein a non-neoplastic neurologic disease, condition, or injury is detected, diagnosed, monitored, prognosticated, or predicted.

64. A diagnostic kit for the detection, diagnosis, monitoring, prognosticating, or predicting of a non-neoplastic disease or pathologic condition or injury of an organ, wherein the diagnostic kit provides for the extraction of RNA from plasma or serum, and provides primers or probes used in the detection of an extracted non-viral mammalian RNA, or cDNA derived therefrom, associated with the diseased or injured organ.

65. The method according to claim 64, wherein the primers or probes hybridize to a mRNA, or cDNA derived therefrom, selected from cardiac troponin T mRNA, cardiac troponin I mRNA, beta-myosin heavy chain mRNA, acidic

fibroblast growth factor mRNA, basic fibroblast growth factor mRNA, or Par-4 mRNA.

66. The method of claim 2, wherein a housekeeper gene RNA is additionally detected.

67. The method of claim 3, wherein a housekeeper gene RNA is additionally amplified and detected.

68. The method of claim 4, wherein a housekeeper gene RNA is additionally amplified and detected.

69. The method of claim 7, wherein a housekeeper gene RNA is additionally amplified and detected.

70. The method of claim 9, wherein a housekeeper gene RNA is additionally amplified and detected.

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(54) Title: METHODS FOR EVALUATING PATHOLOGIC CONDITIONS USING EXTRACELLULAR RNA

(57) Abstract: This invention provides methods for the detection, diagnosing, monitoring or predicting of non-neoplastic diseases, pathologic conditions, and injury. The methods of the invention detect extracellular non-neoplastic mammalian RNA in the blood, blood plasma, serum, or other bodily fluid of an animal, most preferably a human, having or predisposed to having a non-neoplastic disease, pathologic condition, or injury.

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A. CLASSIFICATION OF SUBJECT MATTER

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B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DASI F. et al. Real-Time Quantification in Plasma of Human Telomerase Reverse Transcriptase (hTERT) mRNA: A Simple Blood Test to Monitor Disease In Cancer Patients. Laboratory Investigation. 2001, Vol. 81, No. 5, pages 767-769.	1-70
A	KOPRESKI, M.S. et al. Detection of Tumor Messenger RNA in the Serum of Patients with Malignant Melanoma. Clinical Cancer Research. August 1999, Vol. 5, pages 1961-1965.	1-70

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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